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13. ABSTRACT *(Maximum 200 Words)*

Chromosomal segregation abnormalities are a common feature of breast cancer and several studies estimate that approximately 70% of breast cancers demonstrate aneuploidy. Overall, aneuploid tumors have a worse prognosis compared with diploid tumors. Our laboratory has recently isolated the human homolog of the Rad21 cohesin protein (hRad21) as a protein that interacts with the human Cdc34 ubiquitin-conjugating enzyme. The cohesin proteins are required for proper chromosomal segregation in many eukaryotic organisms. Our work led to the unexpected finding that hRad21 undergoes specific cleavage early in apoptosis. Based on this work, we proposed the novel hypothesis that in mammalian cells chromosomal segregation and the apoptotic cell death pathway are directly linked. In this CONCEPT award we have attempted to test this hypothesis. From these studies, it is apparent that cohesin Rad21 may act as an interface between cohesion and cell death, and its cleavage may signal subsequent events of apoptosis, including DNA degradation. It is likely that hRad21 helps maintain chromosomal stability in mammary cells, and its dysregulation results in aberrant cohesion that leads to aneuploidy. This knowledge will be helpful in the derivation of new strategies for the prevention and treatment of breast cancer.

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INTRODUCTION

Normal development and homeostasis require the orderly regulation of both cell proliferation and cell survival. Cell death by apoptosis plays an essential role in normal development and physiology in the breast [8] as well as in the development of breast cancer[16,18]. The degree of apoptosis can be an important factor in both the progression of breast cancer and the response to treatment[5,10]. A high apoptotic index (number of apoptotic cells per square millimeter of neoplastic tissue) is related to malignant cellular features and is an indicator of invasiveness and cell proliferation in breast cancer[10]. Treatment of breast cancer is improved by increasing the percentage of cells undergoing apoptosis. Thus, cell cycle progression and control of apoptosis are thought to be intimately linked processes. Activation of the cell cycle plays a significant role in the regulation of apoptosis[7]; in some cell types and under certain conditions, apoptosis has been shown to occur only at specific stages of the cell cycle[9]. Although proteins that regulate apoptosis have been implicated in restraining cell cycle entry[6] and controlling ploidy[12], the effector molecules at the interface between cell proliferation and cell survival have remained elusive. **Our aim in this project was to identify the effector molecules that act as a link between cell proliferation, cell survival and chromosome stability. A novel concept initiated in this project is the idea that chromosomal segregation and apoptotic pathways are interlinked and have a role in the development of aneuploidy in breast tumors. The nuclear signal responsible for marking cells destined for programmed death remains a mystery. Some of the untested hypotheses that we set out to explore in this project include whether the nuclear apoptotic signal is linked to sister chromatid cohesion and sister separation during the terminal stages of mitosis, and alternatively, whether proteins involved in apoptosis control cohesion and chromosomal segregation.**

BODY

During the postdoctoral research fellowship funded by the US Army Breast Cancer Research Program (DAMD 17-96-1-6087), the PI isolated the cDNA encoding human Rad21 (Scc1/Mcd1 in budding yeast) in a two-hybrid screen as an interactor of human Cdc34 ubiquitin-conjugating enzyme[14,15]. Analyses of Rad21 function in fission yeast, *S. pombe*, and Scc1/Mcd1 in budding yeast, *S. cerevisiae*, have demonstrated that the nuclear phosphoprotein is required for appropriate chromosomal segregation during the normal mitotic cell cycle and double-strand break repair after DNA damage[3,13]. In budding yeast, loss of cohesion at the metaphase-anaphase transition is accompanied by proteolytic cleavage of the Scc1/Mcd1 protein[17,13], followed by its dissociation from the chromatids[11,13]. Cleavage depends on Esp1 protein (called separin/separase), which is complexed with its inhibitor, Pds1 (also known as securin), before anaphase[4]. In summary, the **Scc1/Mcd1/Rad21 plays a critical role in the eukaryotic cell division cycle by regulating sister chromatid cohesion and separation at the metaphase to anaphase transition**. In mammals, however, little is known about the role of Rad21 cleavage during sister chromatid separation or its impact in human malignancies.

Aim 1) Evaluation of the role of Rad21 in apoptotic response: Initial studies during this project indicated that hRad21 is differentially expressed in a number of breast cancer

derived cell lines in comparison to normal breast epithelial cells. In these studies we examined the expression pattern of Rad21in breast cancer cell lines MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-436, BT-20, HBL100, and SKBR-3 (Fig. 1). In these cells, Rad21 appeared to have altered expression patterns. Compared to normal human mammary epithelial cells (H-MEC), Rad21 mRNA is overexpressed in MDA-MB-231 and SKBR-3. Rad21 protein, however, is found to be overexpressed in all the cells tested, except for BT20, where its expression was found to be considerably downregulated. During these studies we have also generated a number of reagents to study the interaction between hCdc34 and hRad21 and the function of hRad21 in human cell cycle and breast cancer. Several constructs of hRAD21 in myc, flag and HA epitope vectors have been constructed. Rabbit polyclonal antisera using the C-terminal 14 amino acids of hRad21 and a mouse monoclonal antibody to the middle portion of the hRad21 molecule have been raised. Both these antibodies specifically recognize a 120 kDa Rad21 protein in Western blot analyses (Fig.2). Affinity-purified hRad21 polyclonal antibody can efficiently immunoprecipitate the endogenous hRad21 protein (Fig. 2B) from various human and rodent cell lines. Both of the antibodies have also been tested successfully for immunofluorescent detection of endogenous Rad21 protein in the cell.

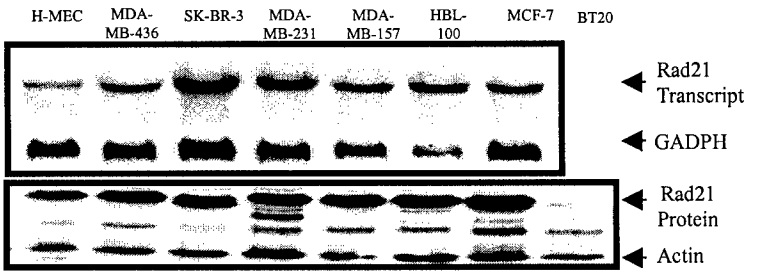


Fig. 1: Expression of hRad21 mRNA and protein in human breast lines. GADPH and Actin are shown as loading control

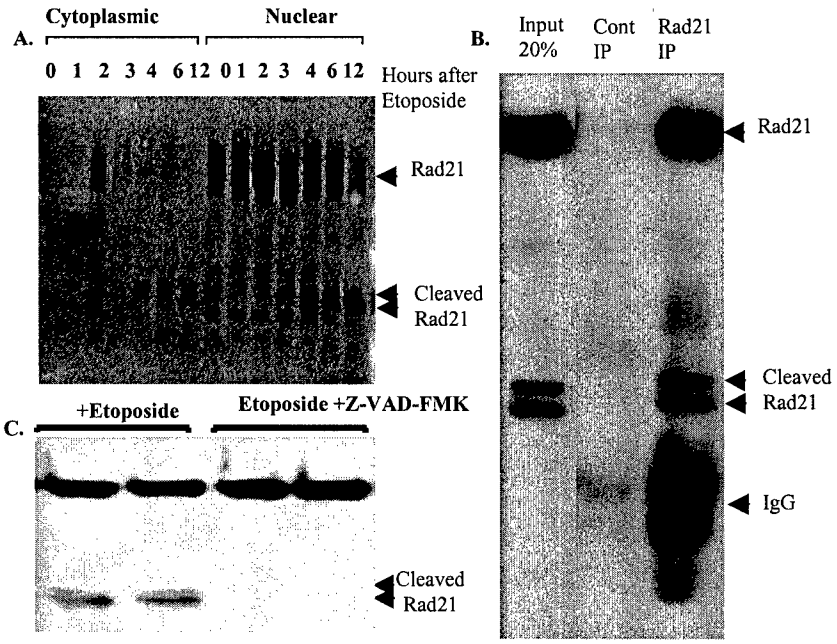


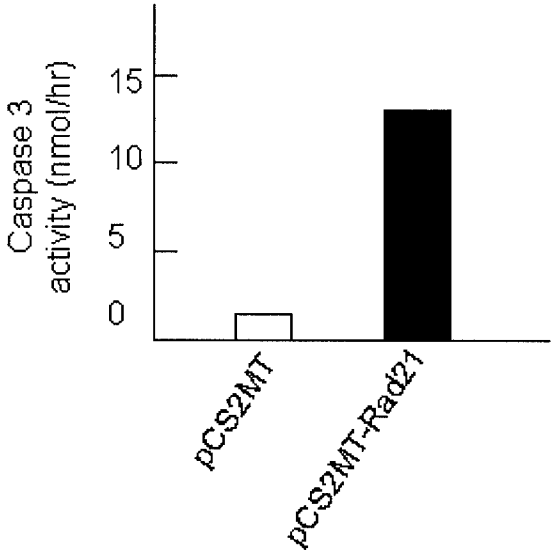
Fig.2**A**: Cleavage of hRad21 and its translocation to cytoplasm after induction of apoptosis in Molt4 T-cells.

B. Immuno-Precipitation and Western blot analysis of cleaved Rad21protein in Molt4 cells treated with 15μM etoposide for 3h.

C. Inhibition of hRad21 cleavage by caspase inhibitor Z-VADFMK.

In a set of studies to analyze the role of Rad21 in mammalian cells after DNA damage, we have recently identified a novel regulatory mechanism of hRad21 in apoptosis. Induction of apoptosis in multiple human cell lines results in the early (3 hours post insult) generation of 64 kDa and

60 kDa cleavage of hRad2 products. Although hRad21 is a nuclear protein, the cleaved product is found in both nuclear and cytoplasmic fractions after the induction of apoptosis (Fig 2A). The identity of this product is confirmed through its recognition by affinity-purified polyclonal and monoclonal antibodies to hRad21 (Fig. 2B) in cell fractionation experiments and immunofluorescent staining assays (data not shown). This product is detected after the induction of apoptosis by DNA-damaging agents (ionizing radiation and topoisomerase inhibitors) as well as non-DNA-damaging agents (cycloheximide treatment and cytokine withdrawal; data not shown). In addition, equivalent doses of ionizing radiation in cells which are resistant to apoptosis do not generate this band; thus, it is not a simple byproduct of the DNA damage. Interestingly, caspase inhibitors block the apoptosis-mediated cleavage of hRad21suggesting the involvement of caspases in Rad21 cleavage (Fig. 2C).



Additional data arguing for an important regulatory role of Rad21 in apoptosis include: 1) overexpression of hRad21 causes apoptosis in human cell lines (fig. 3); 2) Cleaved Rad21 translocates from nucleus to cytoplasm early (~2h) after the induction of apoptosis; 3) A number of genes involved in the apoptosis pathway, including death-associated protein kinase and cytochrome C oxidase, interact with hRad21 in a two-hybrid assay.

Fig. 3. Over-expression of Rad21 tagged from a myc epitope, CMV promoter driven vector (pCS2MT), leads to apoptosis-like phenotypes and enhanced caspase 3 activity in cultured human cells.

Aim 2) Expression of Rad21 Protein and mRNA in breast tumor samples: Expression and localization of the hRad21 protein in normal and malignant breast cancer cells are presently being performed using immunocytochemistry and immunofluorescence techniques. hRad21 efficiently localizes to the nucleus of various mammalian cells assayed by immunoflourescence staining using either the affinity purified polyclonal or monoclonal hRad21 antibodies. Currently, about 250 breast tumor specimens with known status for aneuploidy, estrogen receptor, and a number of other markers with appropriate controls available from the Allred lab at Baylor Breast Center are being used to study the expression and localization of Rad21 protein in these cells using immunohistochemistry [1,2] /immunofluorescence techniques already established in our lab. The Allred lab has assisted us in developing a robust assay to immunolocalize the hRad21 protein using the monoclonal hRad21 antibody (Fig. 4). Since most referral specimens are fixed in 10% neutral buffered formalin and processed at variable rates and

times, increased sensitivity and standardization of the assay is achieved through the use of heat induced antigen retrieval

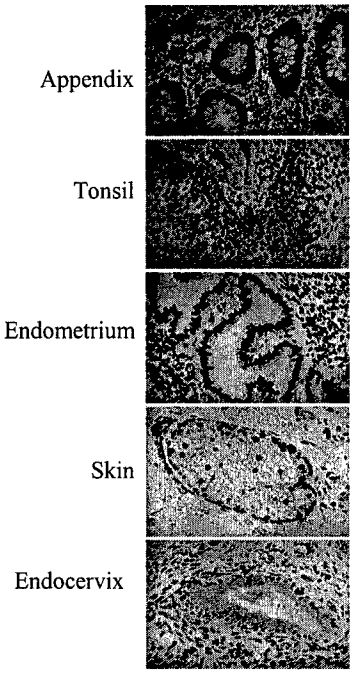


Fig. 4: Development of a immunohistochemical assay for the detection of hRad21 in human tissue sections using a monoclonal hRad21 antibody, *Note the nuclear signal of Rad21 antigen*

post-deparaffinization. Endogenous non-specific protein blocking then follows. Subsequently, incubation with the primary antibody (monoclonal hRad21) is performed, followed by a biotinylated secondary antibody incubation directed against the mouse. These are then followed by incubation in horseradish peroxidase(HRP)-labeled streptavidin, which then binds to the biotin label of the secondary antibody. The entire reaction is then visualized by a incubation with 3,3 diaminobenzedine which, in the presence of HRP, produces a brown reaction product at the site of the antigen-antibody interaction. Enhancement of the reaction product is achieved by the addition of the heavy metal osmium tetroxide, which increases the tone of the reaction product and elevates the signal : noise ratio of the assay. Scoring of immunostained slides for hRad21 expression will be performed according to the protocol recently described for Bcl-2 expression in breast tumor specimens[2] and based on the proportion of cells staining positive.

Due to difficulty in isolating RNA from frozen tumor specimens, expression of *hRAD21* mRNA will be investigated in a subset of breast tumors with Northern blot analysis. The more sensitive RNAse protection assay will be correlated with hRad21 protein levels. Quantitation of *hRAD21* message will be performed using a ^{32}P-labeled probe detected on a Molecular Dynamics STORM imager. Choice of a normal control for expression studies is more difficult. We will use both the MCF-10F cell line and normal human mammary epithelial cells (H-MEC) as controls. In parallel, the expression of hRad21 will be analyzed in the same set of breast tumor specimens using the monoclonal hRad21 antibody previously described. Quantitation will be performed on a Molecular Dynamics STORM imager using the ECL Plus detection system (Amersham). Normalization for loading will be performed by comparing the expression of house-keeping genes such as actin (for protein) and GADPH (for RNA).

Studies in Progress: Currently we have also focused our efforts on mapping the apoptosis-induced Rad21 cleavage sites through protein sequencing. A preliminary set of studies indicated that the expression of the Rad21 protein in specific cellular compartments leads to the induction of apoptosis. Current experiments explore this further by forcing the expression of the full-length as well as the cleaved Rad21 protein into the cytoplasmic compartment using HIV-TAT expression system. The role of Rad21 in apoptosis has been strengthened further by our recent identification of a number of genes in the apoptosis pathway as potential interactors of hRad21 in a two-hybrid assay.

In summary, apart from the previously described functions of Rad21 in chromosome segregation and DNA repair, cleavage of the protein appears to be an early event in the apoptotic pathway. These results provide the framework for identifying importance of Rad21 functions in the apoptotic response of normal and malignant mammary cells with known levels of aneuploidy.

Key Research Accomplishments

- hRad21 is differentially expressed in a number of breast cancer derived cell lines in comparison to normal breast epithelial cells.
- Over-expression of Rad21 in mammalian cells results in the induction of apoptosis.
- Induction of apoptosis by both DNA-damaging and non DNA-damaging agents results in the cleavage of hRad21 protein and cleaved Rad21 protein localized to the cytoplasm. Caspase inhibitors block the apoptosis-induced cleavage of Rad21 protein.
- In a yeast two-hybrid assay a number of genes involved in the apoptosis pathway, including death-associated protein kinase and cytochrome C oxidase, interact with hRad21.

Reportable Outcomes

Abstract:

Pati, D., and Plon, S.E., Cleavage of human Rad21 cohesin protein: potential role in early apoptosis. Abstract book of the Cell Cycle meeting, Cold Spring Harbor Laboratory, New York, May17-21, 2000, p140 (2000)

Fundings received based on work supported by this Award:

US Army IDEA award (DAMD 01-1-0142)
US Army CDA Award (DAMD 01-1-0143)

Employment:

In part, based on the experience supported by this award, PI was promoted to a Tenure Track assistant professor in the Department of Pediatric Hematology/Oncology at Baylor College of Medicine, Houston, TX starting July 2001.

Conclusions

These results have provided the framework for establishing a link between sister chromatid cohesion, the apoptotic response, and the development of aneuploidy, all of which have not previously been tested in any model system. It is apparent that cohesin Rad21 may act as an interface between cohesion and cell death, and its cleavage may signal subsequent events of apoptosis, including DNA degradation. Our on-going experiments, as a part of the IDEA and CDA awards given for the purpose of elucidating the function of cohesin hRad21 in normal and malignant mammary cells, is expected to shed light on the link between cohesion and apoptosis and their importance in the development of anueploidy and malignancies. It is likely that hRad21 helps maintain chromosomal stability in mammary cells, and its dysregulation results in aberrant cohesion that leads to aneuploidy. This knowledge will be helpful in the derivation of new strategies for the prevention and treatment of breast cancer.

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